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(54) Title: NOVEL AMYLOLYTIC ENZYMES DERIVED FROM THE B. LICHENIFORMIS α-AMYLASE, HAVING IMPROVED CHARACTERISTICS

(57) Abstract

    The present invention relates to novel amylolytic enzymes having improved characteristics for the use in starch degradation, in textile or paper desizing and in household detergent compositions. The disclosed α-amylases show surprisingly improved properties with respect to the activity level and the combination of thermostability and a higher activity level. These improved properties make them more suitable for the use under more acidic or more alkaline conditions. The improved properties allow also the reduction of the Calcium concentration under application conditions without a loss of performance of the enzyme.

5

Novel amylolytic enzymes derived from the *B.licheniformis* α-amylase, having improved characteristics

The present invention relates to amylolytic enzymes, particularly α-
10 amylases which are derived from such enzymes as present in *Bacillus licheniformis*.

α-Amylases hydrolyse starch, glycogen and related poly-saccharides by cleaving internal α-1,4-glucosidic bonds at random.

Starch consists of a mixture of amylose (15-30% w/w) and amylopectin
15 (70-85% w/w). Amylose consists of linear chains of α-1,4-linked glucose units having a molecular weight (MW) from about 60,000 to about 800,000. Amylopectin is a branched polymer containing α-1,6 branch points every 24-30 glucose units, its MW may be as high as 100 million.

Starch and especially derivatized starch or thinned starch are important
20 for a number of technical applications, e.g. as substrate for sugar and alcohol production, as an intermediate in polymer production or as technical aid during the production of textiles and paper. Starch is also the major component of stains derived from e.g. chocolate, pap or porridge on clothes and dishes.

25 Thinning of starch, also called liquefaction, is a first step which is necessary in most applications of starch mentioned above. This thinning step can be very conveniently carried out using α-amylase.

The α-amylase used thus far are isolated from a wide variety of bacterial, fungal, plant and animal sources. The industrially most commonly used
30 amylases are those isolated from *Bacilli*.

A known drawback of enzymatic reactions is that enzymes are active over a quite limited range of conditions such as pH, ionic strength and especially temperature.

The α-amylase from *B.licheniformis* is one of the most stable ones in that
35 last respect known so far and is therefore used in applications where the

thermostability of the enzyme is crucial. However, the stability of this enzyme depends on the calcium concentration in the application and the optimum activity is observed at neutral pH. A more thermostable variant of the *B.licheniformis* enzyme, which has the same specific activity as the wild

5 type enzyme, has been described in PCT/EP90/01042.

It has been shown in PCT/DK93/00230 that it is possible to improve the oxidation stability of *B.licheniformis* α-amylase by replacing methionines by one of the other 19 possible amino acids. In the specified test under the given conditions one of these mutants showed a slightly higher activity level

10 than the wild type enzyme.

Though it has been shown that it is possible to improve the stability of amylolytic enzymes, in particular α-amylase, for some detrimental conditions, there is as yet no α-amylase available which has the same or better activity under suboptimal conditions than the wild type enzyme at

15 optimum conditions. Suboptimal conditions are herein defined as conditions which use a pH other than neutral, e.g. lower than 6.5 or higher than 7.5, and/or conditions which use a lower than optimal Ca^{2+} concentration, i.e. lower than 50 ppm.

Because in most industrial applications the conditions are at best

20 suboptimal, the problem of diminished activity could be solved by providing an enzyme which, at optimum conditions, has a higher activity than the wild type enzyme. It would then still have sufficient activity at sub-optimal conditions. The invention provides exactly such enzymes.

The invention provides an amylolytic enzyme derived from the amylolytic

25 enzyme of *Bacillus licheniformis* or an enzyme having at least 70%, or preferably at least 90%, amino acid identity therewith which comprises at least one change of an amino acid in its sequence to another amino acid which provides the enzyme with a higher activity than the wild type enzyme. The activity of an amylolytic enzyme is herein defined as the

30 specific activity as determined in Example 2. The higher activity of the mutant enzymes is apparent under optimal conditions but also under

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suboptimal conditions where a pH value of less than pH 6.5 or higher than pH 7.5 and/or a Ca^{2+} concentration of less then 50 ppm is used. In addition, the invention provides such amylolytic enzymes with a higher thermostability than the wild-type enzyme, wherein the thermostability is defined as determined in Example 3. For some of the mutant enzymes, the improved thermostability is most pronounced under suboptimal conditions regarding the Ca^{2+} concentration.

The amino acid sequence of the *B.licheniformis* α-amylase is shown in Figure 1. The numbers indicate the position of an amino acid in the sequence and will be used as an indication for the amino acid position in the description of the amino acid changes. Regarding the corresponding amino acid changes in enzymes having at least 70%, or preferably at least 90%, amino acid identity with the *B.licheniformis* α-amylase, the skilled person will understand that the *B.licheniformis* α-amylase amino acid positions used herein refer to the corresponding conserved amino acids in the amino acid sequence of these related enzymes and not necessarily to their amino acid positions in those enzymes. It is also to be understood that these corresponding conserved amino acids are not necessarily identical to those of the *B.licheniformis* α-amylase.

In a site directed mutagenesis study we identified mutants on the amino acid sequence which influence the activity level of the enzyme. Among others, we made the following mutations: N104D, S187D, V128E and N188D, which are preferred mutant enzymes according to the invention. Some of these mutants showed a higher overall activity than the wild type enzyme. Alternatively, some of these mutations showed improved thermostability.

Although site directed mutations in the DNA encoding the amylolytic enzymes are a preferred way of arriving at the enzymes according to the invention, the man skilled in the art will be aware that there are different ways of obtaining the enzymes according to the invention and they are therefore part of this invention.

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Due to the fact that until now only 3D-structure of non bacterial α-amylases are available (e.g. L. Brady et al. Acta Cryst. B47 (1991), 527-535, H.J. Swift et al. Acta Cryst. B47 (1991), 535-544, M. Quian et al. J. Mol. Biol. 231 (1993), 785-799), it is hard to predict for the α-amylase from
5 B.licheniformis whether a certain amino acid at a certain position can have any influence on the activity level of the enzyme. One normally needs a 3D-structure for making such predictions, because the spatial orientation of the amino acids determines their role in the catalytic process. Without a 3D-structure of the investigated enzyme one has to relate the results of site
10 directed mutagenesis experiments on putative active site residues on related enzymes (see e.g. L. Holm et al. Protein Engineering 3 (1990) 181-191, M. Vihinen et al. J. Biochem. 107 (1990) 267-272, T. Nagashima et al. Biosci. Biotech. Biochem. 56 (1992) 207-210, K. Takase Eur. J. Biochem. 211 (1993) 899-902, M. Søgaard et al. J. Biol. Chem. 268 (1993) 22480-
15 22484) via a multiple sequence alignment (see e.g. L. Holm et al. Protein Engineering 3 (1990) 181-191) to the known 3D-structures. This allows the identification of the active site residues and allows to identify residues which are conserved in all similar enzymes. One normally assumes that conserved residues are crucial for the function or structure of the enzyme. It
20 is therefore to be expected that mutations in those sites will influence the activity of the enzyme. By making mutations in said active sites it would therefore be expected that some mutations would result in higher activity. However, in B.licheniformis none of the mutated residues at position 104, 128, 187 and 188 are active site residues. Only position 104 is located at
25 the end of a conserved region and could maybe be important for the activity, but also in that particular case a correct prediction of the effect of a point mutation is nearly impossible.

Another important aspect of the invention is the finding that in a number of cases the higher active mutants were slightly less thermostable than the
30 wild type enzyme, except at least the mutations V128E and N188D, which are more stable, or at least more thermostable, than the wild type enzyme.

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We therefore combined them with some earlier identified mutations which are known to stabilize the wild type enzyme. These are the mutations H133Y and T149I. These extra mutations indeed stabilized the more active mutants, but moreover they surprisingly showed an even higher activity level than the higher active mutants themselves.

In a further embodiment of the invention, the mutants of the invention are combined with mutations which improve the oxidation stability of the amylolytic enzyme. Such mutant enzymes may comprise mutations known in the art to improve the oxidation stability of amylolytic enzymes, such as e.g. mutations which replace the methionine at position 197 (see e.g. PCT/DK93/00230).

As stated before, a suitable way of arriving at the enzymes according to the invention is site directed mutagenesis of a nucleic acid, especially a DNA molecule, which comprises the coding sequence for the enzymes. The mutated nucleic acid molecules themselves are also part of the invention representing novel and inventive intermediates in producing the enzymes. Also by providing these nucleic acids in a suitable vector format (whereby a vector is meant to include any suitable vehicle for expression in a cell), it is possible to express the nucleic acid in a vast array of different hosts, including homologous and heterologous hosts, such as bacteria and/or other prokaryotes, yeasts, fungi, plant cells, insect cells or mammalian cells and or other eukaryotic host cells. These host cells which can be cultured to produce the enzymes are also part of the invention.

These cells can be cultured according to known techniques, which are all adapted to the particular kind of cell to be propagated. The isolation of the enzymes according to the invention from the culture or the culture supernatant is also known in the art.

A number of mutants will be more active (i.e. higher specific activity) and/or more stable (with respect to oxidation- and/or thermo-stability) even when only parts thereof are used. These fragments are of course within the

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scope of this invention. It will also be possible to design mutations based upon this invention which have hardly any influence on the activity or stability, such derivatives are also a part of this invention. Some reactive residues which are present in the amino acid sequences according to the invention may also be chemically modified without having significant influence on the activity of such an enzyme. These derivatives are also a part of the invention.

The same may be stated for the nucleic acids according to the invention, which can be modified to a certain extent without influencing the important properties of the resulting enzyme. Therefore nucleic acid sequences which share at least 70% identity, or more preferably at least 90 % identity, with a coding sequence for an enzyme according to the invention or which are complementary to such a sequence are part of this invention. This is also true because based on this invention it will be possible to arrive at similar improvements in activity and/or stability in closely related enzymes such as amylolytic enzymes from *B.stearothermophilus* and *B.amyloliquefaciens*.

The novel amylolytic enzymes according to the invention may be used in all known applications of the amylolytic enzymes in the state of the art.

These applications include the use in the processing of starch, e.g. for polymer production wherein starch needs to be "thinned", the use in detergent compositions to break down stains which comprise starch or starch derivatives, the use in production of sugar or alcohol, or the use in the processing of textile or paper, in particular, the use for desizing of textile or paper, respectively.

Detergent compositions comprising the novel amylolytic enzymes are also a part of the invention. These compositions may be designed for dishwashing (either by hand or automatically), for household or industrial cleaning purposes, or for cleaning textiles. These compositions may comprise the usual additives and/or ingredients such as builders, surfactants, bleaching agents and the like.

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Another preferred embodiment of the invention is the use of the enzymes in producing syrup or isosyrup from starch. Syrup and isosyrup are produced using an α-amylase according to the invention which catalyzes the liquefaction (or thinning) of the starch resulting in dextrins having an average polymerization degree of about 7-10, usually followed by saccharification of the liquefied starch resulting in a syrup with a high glucose content. Optionally the syrup can be isomerized to a dextrose/fructose mixture known as isosyrup.

The invention will now be explained in more detail through the following examples, which are intended for illustration purposes only.

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Examples

Short description of the figures:

Figure 1 gives the amino acid sequence of the α-amylase of *B.licheniformis*. The numbers relate to the positions of the amino acids in the sequence. They are used to identify the mutations, which are given in one letter amino acid code in the text of the application.

The nomenclature used for the mutations is as follows S187D means the replacement of the serine (Ser) at position 187 against an aspartic acid (Asp). Multiple mutants are designated as follows H133Y/T149I means the replacement of histidine (His) at position 133 by tyrosine (Tyr) plus the replacement of threonine (Thr) at position 149 by isoleucine (Ile).

Figure 2 gives a map of plasmid pBHATLAT. α-amylase: *B.licheniformis* α-amylase encoding gene. oripUB: origin of replication of plasmid pUB110. reppUB: replication protein of plasmid pUB110. neo: neomycin resistance gene. bleo: bleomycin resistance gene. pHpaII: HpaII promoter. orifl: origin of replication of phage fl. ori322: origin of replication of plasmid pBR322. bla: ß-lactamase (ampicillin resistance) gene. cat*: inactive chloramphenicol acetyl transferase (chloramphenicol resistance) gene. pTac: Tac promoter.

Example 1
Production and purification of wild type and mutant α-amylases

a)   **Genetic procedures:**

All molecular genetic techniques used for *E.coli* (plasmid construction, transformation, plasmid isolation, etc.) were performed according to

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Maniatis et al. (Molecular Cloning, A Laboratory Manual, Cold Spring Harbor, 1989). Transformation of *B.subtilis* and plasmid isolation were performed according to Harwood et al. (Molecular Biological Methods for Bacillus, Chichester, 1990). *E.coli* strains containing pBHATLAT or its derivatives were grown in the presence of 100 mg/l ampicillin and 2 mg/l neomycin. *Bacillus subtilis* strains harboring pBHLAT 9-derived plasmids were cultivated in medium containing 20 mg/l neomycin.

Plasmid pBHA/C1 is a *Bacillus/E.coli* shuttle vector derived from the twin vector system pMa/c5-8 of Stanssens et al. (Nucl. Acids Res. 17 (1989): 4441-4454). A complete description of pBHA1 is given in the European Patent Application EP 414297.

The *B.licheniformis* α-amylase gene used throughout this study was obtained from plasmid pMcTLia6 (WO91/00353) as an EcoRI-HinDIII restriction fragment still including the inducible Tac promoter. This fragment was inserted in EcoRI-HinDIII digested pBHA1 to yield plasmid pBHATLAT (Fig. 2). This plasmid is used for the expression of α-amylase in *E.coli* through induction of the Tac promoter by 0.2 mM IPTG. Expression of mutant α-amylase was obtained by replacing the wild type α-amylase gene fragment by the corresponding mutant gene fragment. For expression in Bacillus, plasmid pBHATLAT was digested with BamHI and subsequent relegation thus placing the α-amylase gene under the control of the constitutive HpaII promoter. Wild type and mutant α-amylase enzyme was isolated from the Bacillus culture supernatant.

Site directed mutagenesis of the α-amylase gene was performed using the PCR overlap extension technique described by Ho et al. (Gene 77 (1989): 51-59).

b)   Purification of the α-amylase wild type and mutants:

One aliquot of the culture supernatant is added to five aliquots water of 85°C and than maintained at 75°C for 15 minutes. Protease activity is removed in this step. The enzyme is then isolated via ion exchange

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chromatography at pH 5.5 on a S-Sepharose FF column. The buffers used are 20 mM sodium acetate buffer with 1 mM $CaCl_2$ followed, with a gradient, by 20 mM sodium acetate buffer with 1 mM $CaCl_2$ and 0.5 M KCl. The pooled α-amylase fractions are concentrated by ultrafiltration via a 10 kD filter. By washing the concentrate with 1.6 mM EDTA in 50 mM MOPS, pH 7.5 the enzyme can be demetallized. Finally the concentrate is washed twice with 50 mM MOPS buffer pH 7.5.

Example 2
Determination of activity and enzyme concentration

The enzyme concentration is determined by measuring the optical density at 280 nm. The extinction coefficient of wild type enzyme is 135100 M^{-1} cm^{-1}. The mutants with the mutation H133Y have an extinction coefficient of 136430 M^{-1} cm^{-1}. The molecular weight is 55 kD.

The α-Amylase activity is determined by means of the substrate para-Nitrophenyl-maltoheptaosoide (4NP-DP7). The reagent of Abbott (code LN5A23-22) is used. Besides 4NP-DP7 there is also α-glucosidase and glucoamylase in the substrate. α-Amylase activity is measured by the ultimate release of the chromophore p-nitrophenol (pNP).

The terminal glucose unit of the substrate is blocked with a benzylidene group. This terminal blocking inhibits cleavage by α-glucosidase until the initial bonds can be cleaved by α-amylase followed by glucoamylase.

The increase of the OD405 per minute is directly proportional to the α-amylase activity.

The molar extinction coefficient of pNP at 405 nm and pH 6.8 is 7600 M^{-1} cm^{-1}. 1 Unit is 1 μmol converted substrate per minute. With the law "Lambert-Beer" the following relationship is established:

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$$Activity = \frac{OD405 * 10^6}{\epsilon^{405} * l * t} = \frac{OD405}{t} * 131.6 \quad \left[\frac{U}{l}\right]$$

where t = time [minutes], l = lightpath [cm], ϵ^{405} = molar extinction coefficient at 405nm [$M^{-1} * cm^{-1}$], OD405 = extinction at 405 nm, 10^6 = calculation factor from mol/l $\rightarrow$ μmol/l

5   **Activity assay:**

- Add 0.8 ml reagent solution (R1) to a bottle R2 (Abbott).

- Heat the temperature controlled cuvette holder of the spectrophotometer to 37°C.

- Heat the activity buffer to 37°C (50 mM MOPS + 50 mM NaCl + 2 mM
10     $CaCl_2$, pH 6.8).

- Add to the cuvette in the cuvette holder:

   500 μl reagent

   x μl sample

   500 - x μl activity buffer

15   - Measure the increase in extinction at 405 nm during 2 minutes.

- Calculate the activity by using the above equation.

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Table 1

Specific activities of wild type (WT) and mutant α-amylases

Enzyme	Specific Activity [Units/mg]
wild type	60
H133Y	52
H133Y/T149I	60
N104D	30
N104D/H133Y	46
N104D/H133Y/T149I	52
V128E/H133Y	62
V128E/H133Y/T149I	54
S187D	110
H133Y/S187D	155
H133Y/T149I/S187D	150
H133Y/N188D	56
H133Y/T149I/N188D	52
V128E/H133Y/S187D	142

Example 3

Determination of thermostability

The enzyme is incubated in an oil bath at 93 °C in closed Eppendorff micro test tubes with safety lid lock (order-No. 0030 120.086). The Calcium concentration is varied whereas the ionic strength is kept constant. The buffer has at room temperature pH 7.5 which changes at the incubation temperature

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to pH 7.0. A solution of ± 0,25 mg/ml protein in 50 mM MOPS pH 7.5 is obtained by mixing the right amount of enzyme in 50mM MOPS pH 7.5 with **X** mM $CaCl_2$ + **X** mM K_2SO_4 + 100 mM MOPS pH 7.5 + water. The final buffer concentration must be 50 mM and the final volume should be 500 to 1000 μl (the best is 1000 μl). The salt composition is shown on the following table:

mM $CaCl_2$	mM K_2SO_4
0	15
0.25	14.75
0.5	14.5
0.75	14.25
1	14
1.25	13.75
1.5	13.5

Example for 0.5 mM $CaCl_2$:

$\quad\quad\quad\quad$ 250.0 μl 100 mM MOPS pH 7.5

$\quad\quad\quad\quad$ 88.0 μl enzyme (1.42 mg/ml)

$\quad\quad\quad\quad$ 50.0 μl 5 mM $CaCl_2$

$\quad\quad\quad\quad$ 72.5 μl 100 mM K_2SO_4

$\quad\quad\quad\quad$ <u>39.5 μl demi water</u>

$\quad\quad\quad\quad$ 500.0 μl total volume

The enzyme solutions are incubated in the sealed tubes at 93 °C. 50 μl samples are taken after 0.5, 10, 20, 30, 60, 90 and 120 minutes. The residual activity is determined with the Abbott Quickstart Amylase essay (see above). The half life time is calculated by using the fitting program GraFit (Leatherbarrow, R.J. 1990 GraFit version 2.0, Erithacus Software Ltd., Staines, UK).

Table 2

Half life of the WT and mutant α-amylases at different Ca^{2+} concentrations

Ca^{2+}	0	0.25	0.5	0.75	1	1.25	1.5
Enzyme	Half life [min]						
wild type	4.1	9.2	15.5	18.1	22.9	30.3	29.5
H133Y	nd	12.1	24.2	33.3	53.3	nd	77.0
H133Y/T149I	1.1	9.2	21.4	32.8	40.2	53.6	53.6
N104D	nd	nd	nd	nd	7.7	nd	nd
N104D/H133Y	nd	8.4	11.6	nd	14.4	nd	15.4
N104D/H133Y/T149I	nd	10.2	13.4	17.5	19.1	23.1	20.3
V128E/H133Y	nd	15.6	33.9	nd	53.3	65.3	77.8
V128E/H133Y/T149I	nd	19.7	35.2	nd	54.7	nd	76.3
S187D	nd	4.0	6.9	9.3	12.1	nd	15.1
H133Y/S187D	nd	15.2	19.7	27.0	29.8	40.8	47.2
H133Y/T149I/S187D	1.4	6.0	12.7	17.6	20.0	nd	nd
H133Y/N188D	nd	18.2	36.2	nd	70.4	76.8	84.9
H133Y/T149I/N188D	nd	15.8	28.8	nd	62.0	nd	73.6
V128E/H133Y/S187D	1.9	7.2	16.9	nd	32.1	nd	36.2

nd = not determined

Example 4

Starch liquefaction using a mutant α-amylase of the invention

The mutant enzyme was proven to be effective in starch liquefaction tests using industrially relevant conditions. It was tested under identical conditions in comparison with the wild type enzyme.

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A 34.3 % dry solids starch slurry was liquefied using a pilot plant jet cooking apparatus, Hydroheater Model # M 103-MS, at a flow rate of 2.8 l per minute. A 5 minutes retention time at 105 °C of primary liquefaction was followed by a 93 °C 120 min secondary liquefaction. The comparison tests vis a vis the
5 wild type enzyme were performed based upon equal Modified Wohlgemuth Units (MWU) 168 units/gram of starch. The specific activity is for wild type 18,447 MWU/mg and for H133Y/S187D 48,000 MWU/mg respectively.

The enzymes were tested under two sets of conditions. The first experiment used standard industrial conditions (pH 6.4, 44 ppm Calcium),
10 while the second experiment employed stress conditions (pH 5.8, 8 ppm Calcium).

The decrease in viscosity during liquefaction was measured with a #3 Zahn cup, while Dextrose Equivalent (DE) development was measured using a reducing sugar assay. The results are summarized in the following tables:

15

Table 3.

Experiment 1: pH 6.4, 44 ppm Calcium

	Wild type		H133Y/S187D	
time [min]	DE	Viscosity	DE	Viscosity
0		25		24
20	2.7		2.4	
40	4.0		3.5	
60	5.4	14	4.7	14
80	6.5		6.0	
100	7.8		7.5	
120	9.2	12	8.8	12

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Table 4.

Experiment 2: pH 5.8, 8 ppm Calcium

	Wild type		H133Y/S187D	
time [min]	DE	Viscosity	DE	Viscosity
0		36		38
20	0.3		1.1	
40	1.1		2.0	
60	2.0	17	2.9	15
80	2.5		3.5	
100	3.2		4.2	
120	3.9	13	4.6	13

<u>Example 5</u>

<u>Textile desizing using a mutant α-amylase of the invention</u>

Cretonne cotton patches (30 * 30 cm, J. Hacot et Cie., 48 Rue Mermoz, La Gorgue, France) are impregnated with 12 % soluble starch (weight/weight) as sizing agent. The sized cotton is given in a beaker with one litre tap water and 0.5 ml/l wetting agent at 25 °C and pH 7.0. α-Amylase is added in a concentration as shown in the table. The mixture is agitated and heated with a gradient of 2 °C per minute within 30 minutes to a final temperature of 85 °C. After 10 minutes agitating at the final temperature the fabric is 2 minutes rinsed with cold water and dried.

The residual starch is determined with a reflectrometric method. The residual starch on the fibres is coloured with a solution made from 0.15 g iodine, 0.5 g potassium iodine and 10 ml 2 N H_2SO_4 in a volume of 1 l water. The dried cotton patch is wetted with alcohol and soaked in the colouring

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solution for 15 minutes. The reflectance of the coloured patch is measured at 700 nm with a Universal Messeinheit UME 1 III/LR 90 reflectometer (Dr. Bruno Lange GmbH, Berlin, Germany). The amount of residual starch can be calculated with a calibration curve recorded with known amounts of starch on the fabric.

Table 5.

A comparison of the performance of the wild type and a mutant α-amylase in the desizing of textile.

wild type		H133Y/S187D	
enzyme concentration [μmol/l]	remaining starch on fabrics [mg/g]	enzyme concentration [μmol/l]	remaining starch on fabric [mg/g]
0	3.92	0	4.05
9.3	3.35	2.5	3.35
18.5	2.76	4.9	2.45
37.2	2.25	7.6	2.02
46.5	1.85	9.1	1.72
70	1.42	12.5	1.37
93	0.9	18.9	1.12
		25.3	0.68
		37.8	0.5

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Example 6

A comparison of the wash performance of the wild type
and a mutant α-amylase

5   The wash performance of the wild type vis a vis the H133Y/S187D mutant was tested in a full scale wash experiment using the amylase sensitive cotton test fabric EMPA 112 as monitor. In all tests the α-amylase dosage was 1.3 mg/l suds. A blank was taken as reference. Washing powder base was the IEC reference detergent A, containing bleach and protease.

10   All tests were carried out in quintuple. The fabrics were washed in a Miele, type W701 washing machine at 40 °C and a total load of 4 kg fabrics. The soil removal was determined by measuring the white light reflection with a Colorgard Model 05 (Gardner Lab., USA) reflectometer. Table summarizes the results. It shows that the mutant performs better than wild type enzyme at

15   the same dosage.

Table 6.

A comparison of the wash performance of the wild type and a mutant α-amylase

Enzyme	none	wild type	H133Y/S187D
Soil removal	31.7 %	40.2 %	42.1 %

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

5

    (i) APPLICANT:
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10   (E) COUNTRY: The Netherlands
        (F) POSTAL CODE (ZIP): 2611 XT

    (ii) TITLE OF INVENTION: Alpha-amylase mutants

15   (iii) NUMBER OF SEQUENCES: 2

    (iv) COMPUTER READABLE FORM:
        (A) MEDIUM TYPE: Floppy disk
        (B) COMPUTER: IBM PC compatible

20   (C) OPERATING SYSTEM: PC-DOS/MS-DOS
        (D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

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(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 1539 base pairs
    (B) TYPE: nucleic acid
    (C) STRANDEDNESS: double
    (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
    (A) ORGANISM: Bacillus licheniformis
    (B) STRAIN: CBS407.83

(ix) FEATURE:
    (A) NAME/KEY: CDS
    (B) LOCATION: 1..1539

(ix) FEATURE:
    (A) NAME/KEY: sig_peptide
    (B) LOCATION: 1..87

(ix) FEATURE:
    (A) NAME/KEY: mat_peptide
    (B) LOCATION: 88..1539
    (D) OTHER INFORMATION: /product= "alpha-amylase"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

```
ATG AAA CAA CAA AAA CGG CTT TAC GCC CGA TTG CTG ACG CTG TTA TTT      48
Met Lys Gln Gln Lys Arg Leu Tyr Ala Arg Leu Leu Thr Leu Leu Phe
-29              -25                 -20                 -15


GCG CTC ATC TTC TTG CTG CCT CAT TCT GCA GCA GCG GCG GCA AAT CTT      96
Ala Leu Ile Phe Leu Leu Pro His Ser Ala Ala Ala Ala Ala Asn Leu
             -10                 -5                  1


AAT GGG ACG CTG ATG CAG TAT TTT GAA TGG TAC ATG CCC AAT GAC GGC     144
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     Asn Gly Thr Leu Met Gln Tyr Phe Glu Trp Tyr Met Pro Asn Asp Gly
              5                   10                  15

     CAA CAT TGG AAG CGT TTG CAA AAC GAC TCG GCA TAT TTG GCT GAA CAC         192
  5  Gln His Trp Lys Arg Leu Gln Asn Asp Ser Ala Tyr Leu Ala Glu His
          20                  25                  30                  35

     GGT ATT ACT GCC GTC TGG ATT CCC CCG GCA TAT AAG GGA ACT AGT CAA         240
     Gly Ile Thr Ala Val Trp Ile Pro Pro Ala Tyr Lys Gly Thr Ser Gln
 10                      40                  45                  50

     GCG GAT GTG GGC TAC GGT GCT TAC GAC CTT TAT GAT TTA GGG GAG TTT         288
     Ala Asp Val Gly Tyr Gly Ala Tyr Asp Leu Tyr Asp Leu Gly Glu Phe
                      55                  60                  65
 15
     CAT CAA AAA GGG ACG GTT CGG ACA AAG TAC GGC ACA AAA GGA GAG CTG         336
     His Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly Thr Lys Gly Glu Leu
                  70                  75                  80

 20  CAA TCT GCG ATC AAA AGT CTT CAT TCC CGC GAC ATT AAC GTT TAC GGG         384
     Gln Ser Ala Ile Lys Ser Leu His Ser Arg Asp Ile Asn Val Tyr Gly
              85                  90                  95

     GAT GTG GTC ATC AAC CAC AAA GGC GGC GCT GAT GCG ACC GAA GAT GTA         432
 25  Asp Val Val Ile Asn His Lys Gly Gly Ala Asp Ala Thr Glu Asp Val
     100                 105                 110                 115

     ACC GCG GTT GAA GTC GAT CCC GCT GAC CGC AAC CGC GTA ATT TCA GGA         480
     Thr Ala Val Glu Val Asp Pro Ala Asp Arg Asn Arg Val Ile Ser Gly
 30                      120                 125                 130

     GAA CAC CTA ATT AAA GCC TGG ACA CAT TTT CAT TTT CCG GGG CGC GGC         528
     Glu His Leu Ile Lys Ala Trp Thr His Phe His Phe Pro Gly Arg Gly
                      135                 140                 145
 35
     AGC ACA TAC AGC GAT TTT AAA TGG CAT TGG TAC CAT TTT GAC GGA ACC         576
     Ser Thr Tyr Ser Asp Phe Lys Trp His Trp Tyr His Phe Asp Gly Thr
                  150                 155                 160

 40  GAT TGG GAC GAG TCC CGA AAG CTG AAC CGC ATC TAT AAG TTT CAA GGA         624
     Asp Trp Asp Glu Ser Arg Lys Leu Asn Arg Ile Tyr Lys Phe Gln Gly
                  165                 170                 175
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SUBSTITUTE SHEET (RULE 26)

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   AAG GCT TGG GAT TGG GAA GTT TCC AAT GAA AAC GGC AAC TAT GAT TAT        672
   Lys Ala Trp Asp Trp Glu Val Ser Asn Glu Asn Gly Asn Tyr Asp Tyr
   180             185             190             195

5  TTG ATG TAT GCC GAC ATC GAT TAT GAC CAT CCT GAT GTC GCA GCA GAA        720
   Leu Met Tyr Ala Asp Ile Asp Tyr Asp His Pro Asp Val Ala Ala Glu
                   200             205             210

10 ATT AAG AGA TGG GGC ACT TGG TAT GCC AAT GAA CTG CAA TTG GAC GGT        768
   Ile Lys Arg Trp Gly Thr Trp Tyr Ala Asn Glu Leu Gln Leu Asp Gly
               215             220             225

   TTC CGT CTT GAT GCT GTC AAA CAC ATT AAA TTT TCT TTT TTG CGG GAT        816
   Phe Arg Leu Asp Ala Val Lys His Ile Lys Phe Ser Phe Leu Arg Asp
15         230             235             240

   TGG GTT AAT CAT GTC AGG GAA AAA ACG GGG AAG GAA ATG TTT ACG GTA        864
   Trp Val Asn His Val Arg Glu Lys Thr Gly Lys Glu Met Phe Thr Val
         245             250             255

20
   GCT GAA TAT TGG CAG AAT GAC TTG GGC GCC CTG GAA AAC TAT TTG AAC        912
   Ala Glu Tyr Trp Gln Asn Asp Leu Gly Ala Leu Glu Asn Tyr Leu Asn
   260             265             270             275

25 AAA ACA AAT TTT AAT CAT TCA GTG TTT GAC GTG CCG CTT CAT TAT CAG        960
   Lys Thr Asn Phe Asn His Ser Val Phe Asp Val Pro Leu His Tyr Gln
                   280             285             290

   TTC CAT GCT GCA TCG ACA CAG GGA GGC GGC TAT GAT ATG AGG AAA TTG       1008
30 Phe His Ala Ala Ser Thr Gln Gly Gly Gly Tyr Asp Met Arg Lys Leu
               295             300             305

   CTG AAC GGT ACG GTC GTT TCC AAG CAT CCG TTG AAA TCG GTT ACA TTT       1056
   Leu Asn Gly Thr Val Val Ser Lys His Pro Leu Lys Ser Val Thr Phe
35         310             315             320

   GTC GAT AAC CAT GAT ACA CAG CCG GGG CAA TCG CTT GAG TCG ACT GTC       1104
   Val Asp Asn His Asp Thr Gln Pro Gly Gln Ser Leu Glu Ser Thr Val
         325             330             335

40
   CAA ACA TGG TTT AAG CCG CTT GCT TAC GCT TTT ATT CTC ACA AGG GAA       1152
   Gln Thr Trp Phe Lys Pro Leu Ala Tyr Ala Phe Ile Leu Thr Arg Glu
   340             345             350             355
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```
   TCT GGA TAC CCT CAG GTT TTC TAC GGG GAT ATG TAC GGG ACG AAA GGA        1200
   Ser Gly Tyr Pro Gln Val Phe Tyr Gly Asp Met Tyr Gly Thr Lys Gly
                   360                 365                 370

5  GAC TCC CAG CGC GAA ATT CCT GCC TTG AAA CAC AAA ATT GAA CCG ATC        1248
   Asp Ser Gln Arg Glu Ile Pro Ala Leu Lys His Lys Ile Glu Pro Ile
                   375                 380                 385


10 TTA AAA GCG AGA AAA CAG TAT GCG TAC GGA GCA CAG CAT GAT TAT TTC        1296
   Leu Lys Ala Arg Lys Gln Tyr Ala Tyr Gly Ala Gln His Asp Tyr Phe
                   390                 395                 400

   GAC CAC CAT GAC ATT GTC GGC TGG ACA AGG GAA GGC GAC AGC TCG GTT        1344
15 Asp His His Asp Ile Val Gly Trp Thr Arg Glu Gly Asp Ser Ser Val
                   405                 410                 415

   GCA AAT TCA GGT TTG GCG GCA TTA ATA ACA GAC GGA CCC GGT GGG GCA        1392
   Ala Asn Ser Gly Leu Ala Ala Leu Ile Thr Asp Gly Pro Gly Gly Ala
20 420                 425                 430                 435

   AAG CGA ATG TAT GTC GGC CGG CAA AAC GCC GGT GAG ACA TGG CAT GAC        1440
   Lys Arg Met Tyr Val Gly Arg Gln Asn Ala Gly Glu Thr Trp His Asp
                   440                 445                 450
25
   ATT ACC GGA AAC CGT TCG GAG CCG GTT GTC ATC AAT TCG GAA GGC TGG        1488
   Ile Thr Gly Asn Arg Ser Glu Pro Val Val Ile Asn Ser Glu Gly Trp
                   455                 460                 465

30 GGA GAG TTT CAC GTA AAC GGC GGG TCG GTT TCA ATT TAT GTT CAA AGA        1536
   Gly Glu Phe His Val Asn Gly Gly Ser Val Ser Ile Tyr Val Gln Arg
                   470                 475                 480

   TAG
35 1539
```

40 (2) INFORMATION FOR SEQ ID NO: 2:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 512 amino acids

        (B) TYPE: amino acid
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: protein

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Met Lys Gln Gln Lys Arg Leu Tyr Ala Arg Leu Leu Thr Leu Leu Phe
-29   -25   -20   -15

Ala Leu Ile Phe Leu Leu Pro His Ser Ala Ala Ala Ala Ala Asn Leu
        -10   -5   1

Asn Gly Thr Leu Met Gln Tyr Phe Glu Trp Tyr Met Pro Asn Asp Gly
    5   10   15

Gln His Trp Lys Arg Leu Gln Asn Asp Ser Ala Tyr Leu Ala Glu His
20   25   30   35

Gly Ile Thr Ala Val Trp Ile Pro Pro Ala Tyr Lys Gly Thr Ser Gln
            40   45   50

Ala Asp Val Gly Tyr Gly Ala Tyr Asp Leu Tyr Asp Leu Gly Glu Phe
            55   60   65

His Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly Thr Lys Gly Glu Leu
        70   75   80

Gln Ser Ala Ile Lys Ser Leu His Ser Arg Asp Ile Asn Val Tyr Gly
        85   90   95

Asp Val Val Ile Asn His Lys Gly Gly Ala Asp Ala Thr Glu Asp Val
100   105   110   115

Thr Ala Val Glu Val Asp Pro Ala Asp Arg Asn Arg Val Ile Ser Gly
            120   125   130

Glu His Leu Ile Lys Ala Trp Thr His Phe His Phe Pro Gly Arg Gly
            135   140   145

Ser Thr Tyr Ser Asp Phe Lys Trp His Trp Tyr His Phe Asp Gly Thr

```
              150                155                160

    Asp Trp Asp Glu Ser Arg Lys Leu Asn Arg Ile Tyr Lys Phe Gln Gly
        165                170                175
 5
    Lys Ala Trp Asp Trp Glu Val Ser Asn Glu Asn Gly Asn Tyr Asp Tyr
    180                185                190                195

    Leu Met Tyr Ala Asp Ile Asp Tyr Asp His Pro Asp Val Ala Ala Glu
10                  200                205                210

    Ile Lys Arg Trp Gly Thr Trp Tyr Ala Asn Glu Leu Gln Leu Asp Gly
                215                220                225

15  Phe Arg Leu Asp Ala Val Lys His Ile Lys Phe Ser Phe Leu Arg Asp
        230                235                240

    Trp Val Asn His Val Arg Glu Lys Thr Gly Lys Glu Met Phe Thr Val
        245                250                255
20
    Ala Glu Tyr Trp Gln Asn Asp Leu Gly Ala Leu Glu Asn Tyr Leu Asn
    260                265                270                275

    Lys Thr Asn Phe Asn His Ser Val Phe Asp Val Pro Leu His Tyr Gln
25                  280                285                290

    Phe His Ala Ala Ser Thr Gln Gly Gly Gly Tyr Asp Met Arg Lys Leu
                295                300                305

30  Leu Asn Gly Thr Val Val Ser Lys His Pro Leu Lys Ser Val Thr Phe
        310                315                320

    Val Asp Asn His Asp Thr Gln Pro Gly Gln Ser Leu Glu Ser Thr Val
        325                330                335
35
    Gln Thr Trp Phe Lys Pro Leu Ala Tyr Ala Phe Ile Leu Thr Arg Glu
    340                345                350                355

    Ser Gly Tyr Pro Gln Val Phe Tyr Gly Asp Met Tyr Gly Thr Lys Gly
40                  360                365                370

    Asp Ser Gln Arg Glu Ile Pro Ala Leu Lys His Lys Ile Glu Pro Ile
                375                380                385
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```
      Leu Lys Ala Arg Lys Gln Tyr Ala Tyr Gly Ala Gln His Asp Tyr Phe
          390             395             400

      Asp His His Asp Ile Val Gly Trp Thr Arg Glu Gly Asp Ser Ser Val
   5     405             410             415

      Ala Asn Ser Gly Leu Ala Ala Leu Ile Thr Asp Gly Pro Gly Gly Ala
         420             425             430             435

  10  Lys Arg Met Tyr Val Gly Arg Gln Asn Ala Gly Glu Thr Trp His Asp
                     440             445             450

      Ile Thr Gly Asn Arg Ser Glu Pro Val Val Ile Asn Ser Glu Gly Trp
                 455             460             465
  15
      Gly Glu Phe His Val Asn Gly Gly Ser Val Ser Ile Tyr Val Gln Arg
             470             475             480
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Claims

1.   An amylolytic enzyme derived from an α-amylase of *Bacillus licheniformis* or an enzyme having at least 70% amino acid identity therewith which comprises one or more amino acid changes at positions selected from the group consisting of positions 104, 128, 187 and 188 of the amino acid sequence of the α-amylase of *Bacillus licheniformis*.

2.   An enzyme according to claim 1, wherein one or more of the amino acid changes are selected from the group consisting of Asn at position 104 to Asp, Val at position 128 to Glu, Ser at position 187 to Asp, and Asn at position 188 to Asp.

3.   An enzyme according to any one of claims 1 or 2, which comprises at least one additional amino acid change providing the enzyme with improved thermostability.

4.   An enzyme according to claim 3, wherein at least one additional amino acid change selected from the group consisting of His at position 133 to Tyr, and Thr at position 149 to Ile.

5.   An enzyme according to any one of claims 1-4, which comprises at least one additional amino acid change providing the enzyme with improved oxidation stability.

6.   An enzyme according to claim 5, wherein the additional amino acid change comprises a change of a methionine to another amino acid.

7.   An enzyme according to claim 6, wherein the methionine is the methionine at position 197.

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8.   A nucleic acid molecule encoding an enzyme according to any one of the claims 1-7 or a nucleic acid complementary to said nucleic acid or a nucleic acid which hybridizes to either of said nucleic acids under moderately stringent conditions.

9.   A vector for expression of an enzyme according to any one of the claims 1-7, comprising a nucleic acid according to claim 8, together with suitable elements for expression.

10.   A cell for expressing an enzyme according to any one of claims 1-7, comprising a nucleic acid molecule or a vector according to claims 8 or 9, respectively.

11.   A process for producing an enzyme according to any one of claims 1-7, which comprises culturing a cell according to claim 10 in a suitable medium for expression of said enzyme and after a suitable amount of time isolating the enzyme from the culture or the culture supernatant.

12.   Use of an enzyme according to any one of the claim 1-7 in the processing of starch, in the production of syrups, isosyrups, or ethanol, in the desizing of textiles or paper, in brewing processes, in detergents or in the beverage industry.

13.   A detergent composition comprising an enzyme according to any one of claims 1-7.

Figure 1:

```
                    5                   10                  15
     ALA ASN LEU ASN GLY THR LEU MET GLN TYR PHE GLU TRP TYR MET
                    20                  25                  30
     PRO ASN ASP GLY GLN HIS TRP LYS ARG LEU GLN ASN ASP SER ALA
                    35                  40                  45
     TYR LEU ALA GLU HIS GLY ILE THR ALA VAL TRP ILE PRO PRO ALA
                    50                  55                  60
     TYR LYS GLY THR SER GLN ALA ASP VAL GLY TYR GLY ALA TYR ASP
                    65                  70                  75
     LEU TYR ASP LEU GLY GLU PHE HIS GLN LYS GLY THR VAL ARG THR
                    80                  85                  90
     LYS TYR GLY THR LYS GLY GLU LEU GLN SER ALA ILE LYS SER LEU
                    95                  100                 105
     HIS SER ARG ASP ILE ASN VAL TYR GLY ASP VAL VAL ILE ASN HIS
                    110                 115                 120
     LYS GLY GLY ALA ASP ALA THR GLU ASP VAL THR ALA VAL GLU VAL
                    125                 130                 135
     ASP PRO ALA ASP ARG ASN ARG VAL ILE SER GLY GLU HIS LEU ILE
                    140                 145                 150
     LYS ALA TRP THR HIS PHE HIS PHE PRO GLY ARG GLY SER THR TYR
                    155                 160                 165
     SER ASP PHE LYS TRP HIS TRP TYR HIS PHE ASP GLY THR ASP TRP
                    170                 175                 180
     ASP GLU SER ARG LYS LEU ASN ARG ILE TYR LYS PHE GLN GLY LYS
                    185                 190                 195
     ALA TRP ASP TRP GLU VAL SER ASN GLU ASN GLY ASN TYR ASP TYR
                    200                 205                 210
     LEU MET TYR ALA ASP ILE ASP TYR ASP HIS PRO ASP VAL ALA ALA
                    215                 220                 225
     GLU ILE LYS ARG TRP GLY THR TRP TYR ALA ASN GLU LEU GLN LEU
                    230                 235                 240
     ASP GLY PHE ARG LEU ASP ALA VAL LYS HIS ILE LYS PHE SER PHE
                    245                 250                 255
     LEU ARG ASP TRP VAL ASN HIS VAL ARG GLU LYS THR GLY LYS GLU
                    260                 265                 270
     MET PHE THR VAL ALA GLU TYR TRP GLN ASN ASP LEU GLY ALA LEU
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              275                  280                  285
GLU ASN TYR LEU ASN LYS THR ASN PHE ASN HIS SER VAL PHE ASP
              290                  295                  300
VAL PRO LEU HIS TYR GLN PHE HIS ALA ALA SER THR GLN GLY GLY
              305                  310                  315
GLY TYR ASP MET ARG LYS LEU LEU ASN GLY THR VAL VAL SER LYS
              320                  325                  330
HIS PRO LEU LYS SER VAL THR PHE VAL ASP ASN HIS ASP THR GLN
              335                  340                  345
PRO GLY GLN SER LEU GLU SER THR VAL GLN THR TRP PHE LYS PRO
              350                  355                  360
LEU ALA TYR ALA PHE ILE LEU THR ARG GLU SER GLY TYR PRO GLN
              365                  370                  375
VAL PHE TYR GLY ASP MET TYR GLY THR LYS GLY ASP SER GLN ARG
              380                  385                  390
GLU ILE PRO ALA LEU LYS HIS LYS ILE GLU PRO ILE LEU LYS ALA
              495                  400                 ,405
ARG LYS GLN TYR ALA TYR GLY ALA GLN HIS ASP TYR PHE ASP HIS
              410                  415                  420
HIS ASP ILE VAL GLY TRP THR ARG GLU GLY ASP SER SER VAL ALA
              425                  430 ·                435
ASN SER GLY LEU ALA ALA LEU ILE THR ASP GLY PRO GLY GLY ALA
              440                  445                  450
LYS ARG MET TYR VAL GLY ARG GLN ASN ALA GLY GLU THR TRP HIS
              455                  460                  465
ASP ILE THR GLY ASN ARG SER GLU PRO VAL VAL ILE ASN SER GLU
              470                  475                  480
GLY TRP GLY GLU PHE HIS VAL ASN GLY GLY SER VAL SER ILE TYR
              483
    VAL GLN ARG
```

BamHI,1
9140,EcoRI   SpeI,279
                              KpnI,604
8273,ScaI   ClaI,735
           cat*   pTac   NarI,936
                                     SalI,1142
7623,ScaI   alpha-amylase
           bla
                                              HindIII,1848

              pBHATLAT   oripUB

              9242 bps   NcoI,2311

                                         reppUB
            ori322
              orif1
                                      neo
                    pHpaII   bleo
5316,BamHI